Original Article

Endogenous and exogenous galectin-3 promote the adhesion of tumor cells with low expression of MUC1 to HUVECs through upregulating N-cadherin and CD44

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ABSTRACT

Tumor cell-endothelial adhesion is one of the key steps in tumor cell haematogenous dissemination in metastasis and is previously shown to be mediated by interaction of galectin-3 with the transmembrane mucin protein MUC1. In this study, effect of exogenous as well as endogenous galectin-3 on adhesion of low MUC1-expressing human prostate cancer PC-3M cells and non-small cell lung cancer A549 cells to monolayer of umbilical vein endothelial cells (HUVECs) was investigated. It was found suppression of endogenous galectin-3 expression reduced tumor cell adhesion to HUVECs and also decreased cell invasion and migration. Exogenous galectin-3 promoted tumor cell adhesion to HUVECs by entering into cells. Both exogenous and endogenous galectin-3 upregulated the expression of β-catenin and increased β-catenin nuclear accumulation, and then upregulated the expression of N-cadherin and CD44. We deduced that both exogenous as well as endogenous galectin-3 promoted low MUC1-expressing cancer cell adhesion to HUVECs by increasing the expression of N-cadherin and CD44 *via* an increase of β-catenin nuclear accumulation. These results were confirmed further by using β-catenin/TCF transcriptional activity inhibitor, N-cadherin or CD44 siRNAs. This suggests a new molecular mechanism of galectin-3-mediated cell adhesion in cancer metastasis.

KEY WORDS: Galectin-3, Cancer, N-cadherin, CD44, β-catenin, Adhesion

INTRODUCTION

Galectin-3 (Gal-3) is a 29-kDa β-galactoside-binding protein and was firstly identified on murine thioglycollate-elicited peritoneal macrophages.[1] Gal-3 is the only member of chimera type of galectins and is expressed intracellularly and extracellularly by various cell types.[2] It is composed of a carbohydrate recognition domain (CRD) at the C-terminal and a long repetitive collagen-like sequence at the N-terminal domain which is sensitive to proteolysis by MMP-2 and MMP-9.[3] In addition, Gal-3 is able to dimerize and polymerize in some specific conditions, such as at high concentration or the presence of ligands. It is synthesized in the cell cytoplasm and can also be secreted to the cell surface and extracellular environment to be involved in many biological events, such as immune regulation, apoptosis, cell adhesion, mRNA splicing and embryogenesis.

Over the past years, more and more evidence showed that both endogenous and exogenous Gal-3 involved in angiogenesis, cancer cell adhesion, proliferation and metastatic spreading. Endogenous Gal-3 is upregulated in various cancer cells, such as breast cancer, colon cancer, gastric cancer, lung cancer, liver cancer, etc,[4-6] and plays an important role in cell invasion, apoptosis, transcription, mRNA splicing and cell growth. An early research showed that cytoplasmic Gal-3 inhibited apoptosis by binding to B-cell lymphoma 2 (Bcl-2).[7] Endogenous Gal-3 was also shown to increase nuclear β-catenin accumulation by regulation of GSK-3β activity, and promote colon cancer cells progression.[8] The concentrations of Gal-3 in the serum from patients with breast, lung, gastrointestinal, head and neck or ovarian cancer, melanoma and pancreatic cancer were higher than that in serum from healthy individuals. The level of circulating galectin-3 was found apparently higher in the serum of cancer patients those with metastasis.[9] Higher circulating Gal-3 level is considered a potential biomarker for colorectal cancer metastasis.[10] Extracellular Gal-3 is associated with tumor cell dissemination, survival, cancer-matrix adhesion, cancer-endothelial adhesion, angiogenesis and cell growth through interaction with cell surface glycans such as MUC1[11,12] and integrins.[7,13]

Cell adhesion molecules (CAMs) are a group of transmembrane proteins, each of

which is composed of extracellular, transmembrane and cytoplasmic domains. CAMs are divided into five groups including integrins, cadherins, selectins and lymphocyte homing receptors and the immunoglobulin superfamily (Ig-SF). Exogenous Gal-3 was reported to regulate cell adhesion through interaction with N-cadherin, GM1 ganglioside, Mgat5-modified N-glycans, fibronectins, laminin, collagen IV and elastin.[14-18,4] Moreover, Gal-3 was demonstrated to promote polarization of the transmembrane mucin protein MUC1 by binding to the carbohydrate TF (Galβ1,3galNAcα-) antigen. MUC1 polarization by Gal-3 exposes the cadherin molecules, leading to increase cancer cell adhesion to vascular endothelial cells.[11,19] However, effect of extracellular Gal-3 on adhesion of tumor cells with low expression of MUC1 to vascular cells is unknown.

In the present study, we found that endogenous Gal-3 promoted adhesion of tumor cells to HUVECs through upregulation of the expression of CAMs, N-cadherin and CD44, an effect was found to be partly mediated by nuclear accumulation of β-catenin in human non-small cell lung cancer A549 cells. Exogenous introduction of recombinant Gal-3 was also seen to enter into low Gal-3-experssion human prostate cancer PC-3M cells and high Gal-3-expression A549 cells and increase cell β-catenin nucleus accumulation and cell adhesion to HUVECs.

MATERIALS AND METHODS

Materials

RPMI-1640 medium, Ham's F-12K (Kaighn's) medium, fetal bovine serum and L-glutamine were purchased from GINCO BRL (Grand Island, NY, USA). Endothelial cell medium (ECM) were purchased from ScienCell (San Diego, California, USA). Monoclonal antibodies (mAb) against human Gal-3, MMP-2, MMP-9 and β-catenin were purchased from Cell Signaling Technology (Boston, MA, USA). Rabbit Polyclonal antibodies against MUC1, CD44 and N-cadherin were from Proteintech Group, Inc (Chicago, IL, USA). Recombinant full-length human

galectin-3 was from Abcam (Cambridge, UK). ICG-001 was from Aladdin Aladdin Reagent Company (Shanghai, China). ECL reagent, Membrane and Cytosol Protein Extraction Kit and Immunol Fluorence Staining Kit was from Beyotime (Shanghai, China). Subcellular Structure of Cytoplasm and Cell Nucleus Extraction Kit was from Boster Biological Technology (Wuhan, China). Bovine serum albumin (BSA), Vybrant DiO Cell labelling Solutions, radio immunoprecipitation assay (RIPA) lysis buffer and phenylmethane sulfonyl fluoride (PMSF, 100 mmol/L) were purchased from Solarbio (Beijing, China). Gal-3 and N-cadherin siRNAs were purchased from GenePharma Corporation (Shanghai, China). The coding strand targeting by Gal-3 siRNA duplex was 5'-CAC GCT TCA ATG AGA ACA ACA-3' and N-cadherin siRNA duplex was 5'-CTA ACA GGG AGT CAT ATG GTG GAGC-3'. The control siRNA was 5'- TTC TCC GAA CGT GCT GTC TTT-3'. CD44 siRNA was purchased from Santa Cruz Biotechnology (CA, USA). Lipofectamine 2000 Reagent, TRIzol Reagent and Opti-MEM Culture Medium were purchased from Invitrogen (Carlsbad, California, USA). Both MUC1 mRNA and β-actin mRNA were synthesized by GenePharma Corporation (Shanghai, China). qPCR RT Kit and SYBR Green Realtime PCR Master Mix were purchased from TOYOBO (Japan). XenoLight D-Luciferin Potassium Salt was purchased from Caliper Life Sciences (Hopkinton, MA, USA). Culture dish, 6-well plates, 24-well plates and 24-well transwell chambers with 8.0 μm polycarbonated filters were purchased from Corning (NY, USA). Matrigel was purchased from Becton Dickinson and Company (Franklin, USA).

Cell lines

Human prostate carcinoma cell line (PC-3M) was obtained from American Type Tissue Culture (Rockefeller, Maryland, USA). Human non-small cell lung cancer cell line (A549) and Human umbilical vein endothelial cells (HUVECs) were obtained from Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Luciferase-labeled A549 cells (A549-Luc cells) which were kindly provided by

Caliper Life Sciences, Inc (Hopkinton, MA, USA) were cultured in Ham's F-12K (Kaighn's) medium supplemented with 10% fetal bovine serum. A549 cells and PC-3M cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum. HUVECs were cultured in ECM medium supplemented with 10% fetal bovine serum. In addition, 100 U/ml streptomycin / penicillin were added to RPMI 1640 medium and ECM medium. All of the cells were cultured in incubator at 37°C, 5% CO_2.

Gal-3 small interfering RNA (siRNA) transfection

A549 cells (2×10^5) seeded into 6-well plates cultured to 60% confluence before transfection. Transfection with 8 μl control, Gal-3, CD44 or N-cadherin siRNA duplex (25 μmol/L) was performed using 5 μl Lipofectamine 2000 Reagent in 500 μl Opti-MEM Culture Medium. After incubating 8 h, change Opti-MEM Culture Medium to RPMI 1640 medium supplemented with 10% fetal bovine serum. Then cells were incubated at 37°C for 24 h. The efficiency of galectin-3 knockdown was assessed by Western blot analysis of cellular lysates.

Cancer cell-endothelial adhesion

Cancer cell-endothelial adhesion was performed as previously described.[11] Tumor cells were cultured in 6-well plates and HUVECs was cultured in 24-well plates. Tumor cells treated with Gal-3, Gal-3 siRNA (siGal-3) transfection or ICG-001 (β-catenin/TCF transcriptional inhibitor). After washed with PBS, tumor cells were released from the culture plates and labeled with 5 μmol/L DIO fluorescent cell labeling solution in serum-free RPMI 1640 medium for 30 min at 37°C. After resuspension, 5×10^4 cells were applicated to the HUVECs monolayer cultured in 24-well plates for 1 h at 37°C. Then the 24-well plates were gently washed with PBS to remove free tumor cells and the fluorescently labeled cells remaining on the endothelial monolayer were counted in ten randomly selected fields of view using

fluorescent microscope (NIKON Ti-U, Nikon, Japan) with a 10 objective (100 magnifications).

Immunofluorescence staining

A549 and PC-3M cells were seeded into 24-well plates (with coverslips inserted) at density of 5×10^4 cells per well. Gal-3 was added to PC-3M and A549 cells after overnight culture at 37°C. The cells were fixed with ice-cold methanol/glacial acetic acid (3:1) for 10 min and blocked with 3% bovine serum albumin (BSA) in PBS for 20 min at room temperature. Then cells were incubated with a mouse anti-galectin-3 antibody (1:200) overnight at 4°C and followed by reaction with FITC-conjugated secondary antibody (1:500) for 2 h at 37°C. Finally, cells were stained with Hoechst 33342 to stain the nuclear, and fluorescence images were taken with fluorescence microscope (NIKON Ti-U, Nikon, Japan) with a 10 objective (150 magnifications).

Wound scratch assay

A549 cells were seeded into 6-well plates and transfected with siRNA of Gal-3 or treated by ICG-001. The cell monolayer was scraped in a straight line with a p200 pipette tip and incubated under serum-free conditions. Photographs of the scratch were taken under an invert microscope at 0 h, 12 h, 24 h, 36 h and 48 h. Photographs at each time point were taken with Leica DFC420 camera. Gap width analysis was performed with scaleplate in microscope. Measurements were taken at multiple defined sites (>6) along the scratch. Each scratch was given an average of all measurements. Data are derived from three independent experiments.

Formula:

Migration distance = (Gap width at 0 h - Gap width at different time points)/ Gap width at 0 h

Cell invasion assay

A549 cells were seeded into 6-well plates and transfected with siRNA of Gal-3 or

treated by ICG-001. The cells were released from the culture plates and suspended in medium without serum before application of 1×10^6 cells/well to the top side of the transwells. Medium with serum was added to the bottom chamber as a chemoattractant. After incubation at 37°C for 24 h, cells remained in the top chambers (non-invasive) were removed together with the medium. The invaded cells in the bottom chamber were counted under a microscope with a 20 objective (200 magnifications).

Western blot analysis

A549 cells and PC-3M cells were lysed in a RIPA lysis buffer, cytoplasmic extraction reagent and nucleus extraction reagent using Subcellular Structure Cytoplasmic and Nucleus Extraction Kit or membrane extraction reagent and cytoplasmic extraction using Membrane and Cytosol Protein Extraction Kit. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were electrotransferred to PVDF membranes and then blocked with 5% non-fat milk for 4 h. The membranes were then incubated with a primary antibody against galectin-3, β-catenin, MMP-2, MMP-9, CD44, N-cadherin, PCNA and β-actin at 4°C for 12 h. After three washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature and were visualized by ECL reagent through Chemidoc XRS imaging system (Bio-Rad, Hercules, California, USA).

Quantitative real-time PCR

Total RNA was extracted from the cell pellets using TRIzol Reagent following the manufacturer's instructions. The RNA concentration was measured by UV absorption at 260 and 280nm. The A260/A280 ratio was calculated to assess RNA quality and purity. First-strand cDNA was produced from total RNA by using a qPCR RT Kit, according to manufacturer's instructions. Samples were cycled for 30 s at 95°C, 30 s at 59°C and 30 s for 72°C for 40 cycles. QRT-PCR of the MUC1 mRNA and β-actin

mRNA as internal control was performed on a LightCycler 480 II real-time PCR system (Roche, Basel, Switzerland), using the SYBR-Green Chemistry.

Metastasis animal model and bioluminescent imaging

Six-week-old female Balb/c athymic nude mice were maintained and used in accordance with the animal care protocol approved by Shandong University. Ten experimental animals were divided randomly into two groups. 5 animals / group were injected with A549-Luc cells which were transfected with siGal-3 or control siRNA *via* tail vein. The animals were maintained under standard conditions and observed daily. The mice were anesthetized and the fluorescence signal was monitored using the IVIS Kinetic *in vivo* imaging system (Caliper Life Sciences, Hopkinton, MA, USA) in nine minutes after injecting D-luciferin potassium salt intraperitoneally every 10 days for up to 40 days. All mice were sacrificed and dissected for examining metastatic foci by light microscopy at day 40.

Statistical analysis

All quantitative data are expressed as mean ± SD. Statistical comparisons were performed by one-way analysis of variance. A P-value < 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS/Win 13.0 software.

RESULTS

Either endogenous or exogenous Gal-3 increased A549 or PC-3M cells adhesion to HUVECs

Two tumor cell lines, A549 cells which has high expression of endogenous Gal-3[20] or PC-3M cells which has hardly expression of endogenous Gal-3[21] was chose to investigate the effects of endogenous or exogenous Gal-3 on tumor cell-HUVEC adhesion. The expression level of Gal-3 in A549 cells or PC-3M cells was confirmed

in Figure 1A. To detect the effect of endogenous Gal-3 on the adhesion of A549 cells to HUVECs, Gal-3 siRNA was used to down-regulate the expression of Gal-3. A549 cells transfected with Gal-3 siRNA (siGal-3) showed significantly less adhesion (reduction rate of 41.2%, $P < 0.01$) to HUVECs compared with A549 cells treated with control siRNA (Fig. 1E).

It was reported previously that exogenous Gal-3 at concentrations similar to that seen in cancer patients promoted cancer cell adhesion to HUVECs by interaction with MUC1 expressed on the membrane of cancer cells.[11,19] However, in the present study, we want to observe the effects of exogenous Gal-3 on the adhesion of HUVECs to cancer cells with low expression of MUC1. A549 and PC-3M cells were reported to have low MUC1 expression,[22,23] which was confirmed in Figure B using HT-29 cells as positive control cell lines with high expression of MUC1. What's more, MUC1 mRNA expression in A549 and PC-3M cells was also lower than that in HT-29 cells (Fig. 1C). In addition, the recombinant Gal-3 could not affect the expression of MUC1 in these two cell lines (Fig. 1D). We examined the adhesion of A549 or PC-3M cells to HUVECs by incubation with different concentration of recombinant Gal-3 for 8 h. Results showed that 0.1, 0.5, 1 μg/ml Gal-3 significantly increased PC-3M cells adhesion to HUVECs by 12.6%, 33.7% and 56%, and increased A549 cell adhesion to HUVECs by 19.6%, 39.4% and 55.1% compared with control cells (Fig. 1F and 1G). These results indicated exogenous Gal-3 may promote tumor cell-HUVEC adhesion also through a MUC1-independent mechanism.

Endogenous Gal-3 promoted A549 cells-HUVECs adhesion by upregulating CD44 and N-cadherin expression

Previous studies showed that endogenous Gal-3 could bind to β-catenin and regulate accumulation of β-catenin in nucleus.[8] In this study, downregulation of Gal-3 expression in A549 cells was shown to be associated with reduction of cell adhesion and decrease of the accumulation of β-catenin in nucleus (Fig. 2A).

To investigate whether β-catenin was involved in A549 cells adhesion to HUVECs, ICG-001, a small-molecule inhibitor to specially block β-catenin/TCF transcriptional

activity,[24] was introduced into the adhesion system. It was found that pretreatment of A549 cells with 5 μmol/L and 10 μmol/L ICG-001 resulted in reduction of A549 cells adhesion to HUVECs with reduction rate of 11.4% ($P < 0.05$) and 42.1% ($P < 0.01$), respectively compared with untreated A549 cells (Fig. 2B).

To further determine whether the effect of endogenous Gal-3 on cancer cell-endothelial adhesion was involved in β-catenin/TCF transcriptional activity, A549 cells were incubated with ICG-001 after being transfected with control siRNA or siGal-3. As shown in Fig. 2C, in the presence of ICG-001 (10 μmol/L), control siRNA and siGal-3 treatment induced similar effect on reduction (51.2%, $P < 0.01$ and 49.3%, $P < 0.01$) of A549 cells adhesion to HUVECs compared to blank control group. These data indicated that the involvement of endogenous Gal-3 in the adhesion of A549 cells to HUVECs was depending on β-catenin/TCF transcriptional activity.

As many CAMs are involved in mediating cancer cell-endothelial adhesion and CD44 was regulated by β-catenin,[25] we speculated that endogenous Gal-3 might promote A549 cells adhesion to HUVECs through regulating the expression of N-cadherin and CD44 *via* β-catenin/TCF. High expression of N-cadherin showed in both HUVECs and A549 cells (Fig. 3B) which suggested that N-cadherin might be a key CAM involving in A549 cell-HUVEC adhesion. As shown in Fig. 3A, downregulation of cell Gal-3 expression inhibited the expression of N-cadherin and CD44 in A549 cells. After treatment with ICG-001 (β-catenin/TCF transcriptional inhibitor), the expression of N-cadherin in A549 cells was decreased in a concentration-dependent manner, while the expression of CD44 showed no significant change which suggested that CD44 is also regulated by other ways (Fig. 3C). However, in the presence of 10 μmol/L ICG-001, Gal-3 downregulation did not decrease the expression of N-cadherin and CD44 (Fig. 3D). These indicated that the decreased expressions of N-cadherin and CD44 induced by Gal-3 downregulation were dependent on the presence of β-catenin.

Recombinant Gal-3 promoted tumor cell (low MUC1 expression) adhesion to HUVECs by entering into cells and upregulating β-catenin expression and

nuclear accumulation

We next explored the mechanism of exogenous Gal-3 on MUC1-independent cancer cells adhesion to HUVECs. It has been reported that exogenous Gal-3 had the ability to enter macrophages *via* endocytosis.[26] Thus, we speculated that introduction of recombinant Gal-3 to the cells could get into the cells and regulate adhesion of A549 or PC-3M cells (with low MUC1 expression) to HUVECs through the similar ways as intracellular Gal-3. Firstly, recombinant Gal-3 showed the ability to enter into no gal-3 expressing PC-3M cells (Fig. 4A). After incubation with recombinant Gal-3, the expression of Gal-3 in PC-3M cells was rapidly increased in a time-dependent manner during 2 h, and then decreased gradually within 8 h. After incubation of PC-3M cells with 0.5, 1 μg/ml recombinant Gal-3 for 8 h, Gal-3 entered into both cytoplasm and nucleus in dose-dependent manner (Fig. 4B, 4C). Recombinant Gal-3 also showed ability to enter into A549 cells (with high expression of endogenous Gal-3) after down-regulating the expression of Gal-3 by RNA interference (siGal-3) (Fig. 5A). Results showed that Gal-3 could be seen in both cytoplasm and nucleus of A549 cells in endogenous Gal-3 knockdown cells (Fig. 5B). In order to rule out cell membrane contamination of Gal-3 expression in cytoplasm, we separated the cell membrane and cytoplasm using Membrane and Cytosol Protein Extraction Kit. The result showed the expression of Gal-3 in cytoplasm was significantly increased after incubation of PC-3M and A549 cells with 1 μg/ml recombinant Gal-3 for 2 h (Fig. 5C). So, the present data confirmed that exogenous recombinant Gal-3 had the ability to enter the cytoplasm and nucleus of tumor cells with low level of endogenous Gal-3. Simultaneously, the entrance of Gal-3 in PC-3M cells was accompanied with the increased expression of β-catenin in nucleus which indicated its nucleus accumulation (Fig. 4C). Moreover, the expression of β-catenin in both cytoplasm and nucleus in A549 cells transfected with siGal-3 also decreased, while it was increased in the presence of recombinant Gal-3 (Fig. 5B).

After being transfected with siGal-3, the adhesion of A549 cells to HUVECs was significantly increased by introduction of 1 μg/ml recombinant Gal-3 for 8 h (Fig. 6A). But this effect did not occur in the presence of 10 μmol/L ICG-001 (Fig. 6B).

Similarly, the presence of 10 μmol/L ICG-001 reduced recombinant Gal-3 (1 μg/ml, 8 h)-mediated increase of PC-3M cell adhesion to HUVECs (Fig. 6C).

Taken together, these results indicated that exogenous Gal-3 could promote the adhesion to HUVECs of tumor cells with low MUC1-expression by upregulating β-catenin and its nuclear translocation.

N-cadherin and CD44 were involved in promoting tumor cells adhesion to HUVECs induced by exogenous Gal-3

After treatment of PC-3M cells with 1 μg/ml recombinant Gal-3 for 0 h, 1 h, 2 h, 4 h and 8 h, the expression of N-cadherin and CD44 in PC-3M cells showed a time-dependent increase (Fig. 4B). After treatment with 0.5, 1 μg/ml recombinant Gal-3 for 8 h, the expressions of N-cadherin and CD44 in PC-3M cells or A549 cells were significantly increased (Fig. 7A and 7B). Moreover, after A549 cells transfected with Gal-3 siRNA, the endogenous expression of N-cadherin and CD44 decreased in comparison with that in control siRNA cells, while increased after incubation with recombinant Gal-3 for 8 h (Fig. 7C). To further investigate the relationship between increased expression of N-cadherin and CD44 induced by recombinant Gal-3 and β-catenin, 10 μmol/L ICG-001 was used to inhibit the β-catenin/TCF transcriptional activity.

However, when A549 cells were exposed to ICG-001 (10 μmol/L), the expression of N-cadherin significantly decreased while the expression of CD44 has no markedly difference. And recombination Gal-3 couldn’t alter the expression of these two CAMs in the presence of ICG-001 (Fig. 7D). These results suggested that exogenous Gal-3 could upregulate the expression of N-cadherin and CD44 in both A549 cells and PC-3M cells in the presence of β-catenin.

To gain further insight into the role of N-cadherin and CD44 in regulating adhesion of tumor cells to HUVECs, siRNA to N-cadherin or CD44 in A549 cells was conducted before performance of cell adhesion to HUVECs. It was found that suppression of N-cadherin or CD44 by RNA interference resulted in reduction of adhesion of A549 cells to HUVECs. After treatment with 1 μg/ml recombinant Gal-3

for 8 h, adhesion of A549 cells transfected with siCD44 or siN-cadherin to HUVECs was significantly increased, accompanying with an increase of CD44 and N-cadherin expression (Fig. 8A, 8B, 8C). Interestingly, suppression of N-cadherin expression induced more reduction of A549 cells adhesion to HUVECs (53.7%) than suppression of CD44 expression (36.3%). These results indicate that N-cadherin and CD44 may both play an essential role in Gal-3-mediated tumor cell-HUVEC adhesion, but N-cadherin may make more contribution than CD44.

Endogenous Gal-3 promoted migration and invasion of A549 cells also depending on β-catenin/TCF transcriptional activity

In the wound scratch assay, suppression of Gal-3 expression by RNA interference in A549 cells resulted in slower closure of the gaps than that in blank group or control siRNA group for 0 h, 12 h, 24 h, 36 h and 48 h. In the presence of ICG-001 (10 μmol/L), the A549 cell migration was significantly inhibited at 24 h, 36 h and 48 h in comparison to blank group. Moreover, co-presence of siGal-3 and ICG-001 almost completely abolished the ability of A549 cell migration (Fig. 9A).

In the invasion assay, the number of invasive A549 cells through matrigel was decreased significantly by suppression of Gal-3 expression or ICG-001 in compared with control siRNA or blank group. Co-presence of siGal-3 and ICG-001 dramatically inhibited the invasion of A549 cells (Fig. 9B). Moreover, we also observed that suppression of Gal-3 expression decreased the expression of β-catenin in both cytoplasm and nucleus (Fig. 5B). These results indicate that endogenous Gal-3 promotes the expression of β-catenin in cytoplasm and its nucleus accumulation, which may play a crucial role in A549 cells migration and invasion. Moreover, the expression of MMP-2 and MMP-9 in A549 cells was inhibited by the presence of ICG-001 in a concentration-dependent manner, indicating that both MMP-2 and MMP-9 may contribute to the migration and invasion ability of A549 cells induced by Gal-3/β-catenin pathway (Fig. 9C).

Downregulation of galectin-3 expression inhibited the metastasis of A549 cells *in*

vivo

To explore the effects of Gal-3 in tumor metastasis *in vivo*, 10 nude mice developed metastasis after injection of A549-Luc cells which were transfected with siGal-3 or control siRNA *via* tail vein. The lung metastasis of A549-Luc cells in nude mice at different time points was observed using *in vivo* imaging system. At day 10, obvious metastatic foci in the lungs appeared in all mice injected with A459-Luc cells by luciferase image, while weak foci showed in only one mouse injected with A549-Luc cells transfected with siGal-3 (Fig. 10A). At either day 20 or day 40, the luminescence intensity of metastatic foci in the lungs in mice injected with A459-Luc cells transfected with siGal-3 were significantly less than that in mice injected with A549-Luc cells (Fig. 10B and 10C). The macroscopic appearance of lungs at 40d in two mice groups also showed the same results as luminescence intensity (Fig. 10D).. These results indicated that pretreatment of A549 cells with siGal-3 transfection resulted in an obvious inhibition on the pulmonary metastasis of A549 cells.

DISCUSSION

Gal-3 is a β-galactoside-binding protein that located inside and outside cells, and is widely expressed in various tissues. Gal-3 interacts with glycoproteins expressed on cell surface through its CRD. Gal-3 can bind to β-galactosides present in N-glycans and O-glycans.[27] Thus, Gal-3 could interact with the glycoprotein MUC1 and EGFR, then regulated MUC1 and EGFR cellular distribution and activated EGFR downstream pathways in pancreatic cancer cells.[28] Exogenous Gal-3 was shown to promote tumor cell homotypic aggregation and adhesion to HUVECs and increase EGFR dimerization and activation[29] by interaction with O-linked glycans on MUC1.[12,19,30] In this study, exogenous and endogenous galectin-3 showed regulation on adhesion of tumor cells through MUC1-independent mechanisms.

Firstly, we found that suppression of endogenous Gal-3 by siGal-3 in A549 cells decreased cell adhesion to HUVECs, an effect that is associated with downregulation of the expressions of β-catenin and two CAMs, N-cadherin and CD44. Adhesion of A549 cells to HUVECs was seen to be significantly decreased, and the expression of

N-cadherin was decreased while the expression of CD44 was not affected in the presence of β-catenin/TCF-mediated transcription inhibitor ICG-001. This indicates that β-catenin/TCF-mediated transcriptional activity was closely associated with A549 cell adhesion to HUVECs and the expression of N-cadherin. The presence of ICG-001 showed to abolish Gal-3-mediated increase of A549 adhesion to HUVEC. We therefore deduce that endogenous Gal-3 promotes A549 cells adhesion to HUVECs through upregulation of N-cadherin and CD44 *via* β-catenin/TCF transcription.

The Wnt/β-catenin signaling pathway plays an essential role in homeostasis, organogenesis and cancer progression. In the absence of Wnt stimuli, β-catenin is degraded by the complex that contains axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK-3β). Aberrant activation of the Wnt/β-catenin pathway can increase β-catenin accumulation in nucleus, leading to activation of the transcription of Wnt-target genes including cyclin D1, c-Myc.[31] The expression of N-cadherin was upregulated in many tumors, such as prostate cancer, breast cancer, gastric cancer, colon cancer, pancreatic cancer, esophagus cancer and melanoma cancer.[32-38] In addition, expression of N-cadherin enhances the movement of HCT-8/E11 colon cancer cells[39,40] and was involved in stromal-mesenchymal cell adhesion.[41] In the present study, the high expression of N-cadherin in HUVECs and A549 cells demonstrated that N-cadherin might be a key CAM to mediate the adhesion of these two cells. N-cadherin also showed to increase breast cancer adhesion to endothelial cells and enhance tumor cell ability to enter and exit the blood vessel.[42] N-cadherin was also reported to increase expression of MMP-9 by activating MEK/MAPK pathway, eventually promote cancer metastasis and invasion.[43] It was previously reported that N-cadherin was upregulated in epithelial-mesenchymal transition (EMT)[36,44] which could be induced by activated β-catenin/LEF-1 signaling pathway directly.[45] However, the transcriptional mechanism of N-cadherin remains unclear. In this study, Gal-3 expression suppression decreased β-catenin expression both in cytoplasm and in nucleus, indicating that endogenous Gal-3 promoted nuclear accumulation of β-catenin. This result was in consistent with early studies.[5,46] N-cadherin expression was downregulated by either endogenous Gal-3 suppression or

exposure to β-catenin/TCF-mediated transcription inhibitor ICG-001 in A549 cells. Furthermore, endogenous Gal-3 suppression couldn't downregulate the expression of N-cadherin in the presence of ICG-001. These results indicate that endogenous Gal-3 upregulates the expression of N-cadherin through promotion of nuclear translocation of β-catenin.

CD44 is a transmembrane glycoprotein expressed on various cell surfaces[47] and plays a crucial role in regulating adhesion of cell-cell and cell-ECM.[48] It promotes cancer metastasis in many cancers such as breast cancer, gastric cancer, colon cancer, cervical cancer.[49-52] In addition, CD44 has been reported to be a target protein of Wnt/β-catenin pathway.[51,53] In this study, suppression of endogenous Gal-3 expression was seen to decrease the expression of CD44, which was abolished by ICG-001. These indicated that the decreased expressions of CD44 induced by Gal-3 suppression were dependent on the presence of β-catenin. However, the expression of CD44 showed no significant change after treatment with ICG-001 to inhibit the activity of β-catenin/TCF-mediated transcription, suggesting that there may be other regulatory mechanism of CD44 transcription which is β-catenin-independent.

In this study, endogenous Gal-3 was observed to promote migration and invasion of A549 cells depending on β-catenin/TCF transcriptional activity. A combined treatment of the cells with ICG-001 and siGal-3 produced a synergistic inhibition on A549 cell invasion and migration. This implied that β-catenin/TCF transcriptional activity played an important role in Gal-3 induced migration and invasion. Both N-cadherin and CD44 have been shown previously to promote tumor cell migration and invasion,[32-35,38,50,54] which was in consistence with the results shown in this study. The discovery that ICG-001 also inhibited the expression of MMP-9 and MMP-2 suggests that these MMP members may contribute to the migration and invasion of A549 cells induced by Gal-3/β-catenin pathway.

Previous study had shown that exogenous Gal-3 increased tumor cells adhesion to HUVECs by binding to MUC1 on cell surface to expose small CAMs.[11] In this study, we observed the effect of recombinant Gal-3 on low MUC1 expressing PC-3M cells and A549 cells adhesion to HUVECs. Results showed that recombinant Gal-3 also

increased low MUC1 expressing tumor cells adhesion to HUVECs, suggesting that other mechanisms being independent on MUC1 existed. An early study reported that exogenous Gal-3 could be rapidly internalized from the exogenous compartment by endocytosis in macrophages.[26] So we supposed that recombinant Gal-3 could be internalized in tumor cells *via* endocytosis. The current results verified this assumption that Gal-3 entered into the cytoplasm and nucleus of PC-3M cells and A549 cells by separating cytoplasm with nucleus and cytomembrane with cytoplasm. Furthermore, results showed that β-catenin was increased in cytoplasm and nucleus of PC-3M cells and A549 cells after incubation with recombinant Gal-3. Once β-catenin/TCF-mediated transcription was inhibited by the presence of ICG-001, the increase of recombinant galectin-3-mediated cell adhesion was reduced. These indicated that exogenous Gal-3 upregulated cancer cells adhesion to HUVECs also partly depending on nucleus accumulation of β-catenin as the same as endogenous Gal-3. In addition, recombinant Gal-3 significantly increased the expression of N-cadherin and CD44 in either PC-3M cells or A549 cells, while could not regulate the expression of these two CAMs in the presence of ICG-001, suggesting that exogenous Gal-3 upregulated the expression of N-cadherin and CD44 in both A549 cells and PC-3M cells through promoting β-catenin/TCF-mediating transcription. What's more, A549 cells adhesion to HUVECs was inhibited by suppression of N-cadherin and CD44, increased after treatment with recombinant Gal-3 by upregulating the expression of these two CAMs, further indicating that exogenous Gal-3 promoted cancer cells adhesion to HUVECs by increasing the expression of N-cadherin and CD44 in the presence of β-catenin.

In conclusion, exogenous as well as endogenous Gal-3 promotes cancer cells adhesion to vascular endothelial cells by increasing the expression of N-cadherin and CD44 *via* an increase of β-catenin nuclear accumulation. Moreover, the effect of endogenous Gal-3 on the metastasis of A549 cells with low expression of MUC1 was confirmed *in vivo*. This indicates a new molecular mechanism of Gal-3-mediated cell adhesion in cancer metastasis and may have therapeutic potential for developing new strategies to prevent metastasis.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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Figure legends

Figure 1. Effects of intracellular and extracellular Gal-3 on tumor cells adhesion to HUVECs. Tumor cells adhesion to HUVEC monolayer was assessed using DIO fluorescent-labelled tumor cells. Expression of Ga1-3 (A) and MUC1 (B) in HT-29, A549 or PC-3M cells was determined by Western blotting. C: MUC1 mRNA levels in HT-29, A549 and PC-3M cells were determined by qRT-PCR. D: Recombinant Gal-3 could not affect the expression of MUC1 in A549 and PC-3M cells. E: siRNA Gal-3 suppression decreased A549 cells adhesion to HUVECs. The adhesion of PC-3M cells (F) or A549 cells (G) to HUVECs was determined after treatment of the cells with different concentration of recombinant Gal-3 for 8 h. Data are presented as the means ± S.E. from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS, not significant, when compared with blank group.

Figure 2. Downregulation of Gal-3 expression decreased A549 cell adhesion to HUVECs through inhibition of nuclear accumulation of β-catenin. A: suppression of cell-associated Gal-3 significantly inhibited accumulation of β-catenin in nucleus. B: ICG-001 decreased A549 cell adhesion to HUVECs. C: ICG-001 abolished inhibition of A549 cell adhesion to HUVECs induced by suppression of cell-associated Gal-3. Data are presented as the means ± S.E. from three separate experiments. $^{*}P < 0.05$, $^{***}P < 0.001$, NS, not significant, *vs*. blank group.

Figure 3. Downregulation of N-cadherin and CD44 expression induced by suppression of cell-associated Gal-3 was abolished by inhibition of β-catenin/TCF gene transcription. A: The expression of N-cadherin, CD44 in A549 cells was downregulated by siGal-3 treatment. B: High expression of N-cadherin showed in either HUVECs or A549 cells. C: ICG-001 decreased the expression of N-cadherin but not CD44. D: ICG-001 abolished the downregulation of N-cadherin and CD44 induced by suppression of cell-associated Gal-3. Data are presented as the means ± S.E. from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, *vs*. control group.

Figure 4. Exogenous Gal-3 increased nuclear accumulation of β-catenin *via* internalization in PC-3M cells. A: PC-3M cells were treated with 1 μg/ml Gal-3 for 2 h. Gal-3 was visualized with an anti-galectin-3 antiboby (red), and the cell nucleus was visualized with Hoechst 33342 (bule). B: The expression of Gal-3, N-cadherin and CD44 in PC-3M cells after incubation with 0.5 μg/ml recombinant Gal-3 at different time points was assessed by Western blotting. C: After treatment with 0.5, 1 μg/ml Gal-3 for 8 h, the expression of Gal-3 and β-catenin in cytoplasm and nucleus of PC-3M cells was assessed by Subcellular Structure of Cytoplasm and Cell Nucleus Extraction Kit and Western blotting. The band densities were quantified by Chemidoc XRS imaging system (Bio-Rad, Hercules, California, USA) and were normalized to β-actin. Data are presented as the means ± S.E. from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *vs*. control group.

Figure 5. Exogenous Gal-3 increased nuclear accumulation of β-catenin *via* internalization in A549 cells. A: A549 cells were treated with 1 μg/ml Gal-3 for 2 h. Gal-3 was visualized with an anti-galectin-3 antiboby (red), and the cell nucleus was visualized with Hoechst 33342 (bule). B: After siRNA suppression of Gal-3, A549 cells were incubated with or without 1 μg/ml Gal-3 for 8 h, and the expression of gal-3 and β-catenin in cytoplasm and nucleus of A549 cells was assessed by Subcellular Structure of Cytoplasm and Cell Nucleus Extraction Kit and Western blotting. C: After treatment with 1 μg/ml Gal-3 for 2 h, the expression of Gal-3 in cytoplasm and cytomembrane of A549 and PC-3M cells was assessed by Membrane and Cytosol Protein Extraction Kit and Western blotting. The band densities were quantified by Chemidoc XRS imaging system (Bio-Rad, Hercules, California, USA) and were normalized to β-actin. Data are presented as the means ± S.E. from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *vs*. control group.

Figure 6. Effect of exogenous Gal-3 on A549 cell adhesion to HUVECs was affected by siGal-3 and ICG-001. A: Exogenous Gal-3 increases adhesion of A549 cells which was transfected with siGal-3. B: The increase of Gal-3-mediated A549 cell adhesion

to HUVECs was significantly inhibited by the presence of ICG-001. C: The increase of Gal-3-mediated PC-3M cell adhesion to HUVECs was significantly inhibited by the presence of ICG-001. Data are presented as the means ± S.E. from three separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, NS, not significant.

Figure 7. Exogenous Gal-3 increased the expression of N-cadherin and CD44 in PC-3M and A549 cells by internalization. A and B: After 8 h treatment of the cells with recombination Gal-3 (0.5, 1 μg/ml) expression of N-cadherin and CD44 in PC-3M cells (A) and A549 cells (B) were both increased. C and D: The expression of N-cadherin, CD44 and Gal-3 were assessed after incubation of the Gal-3 suppressed A549 cells with or without 1 μg/ml Gal-3 for 8 h (C) or in the presence of 10 μmol/L ICG-001 (D). The band densities of these proteins were quantified by Chemidoc XRS imaging system (Bio-Rad, Hercules, California, USA) and normalized to β-actin. Data are presented as the means ± S.E. from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, *vs*. control group. NS, not significant.

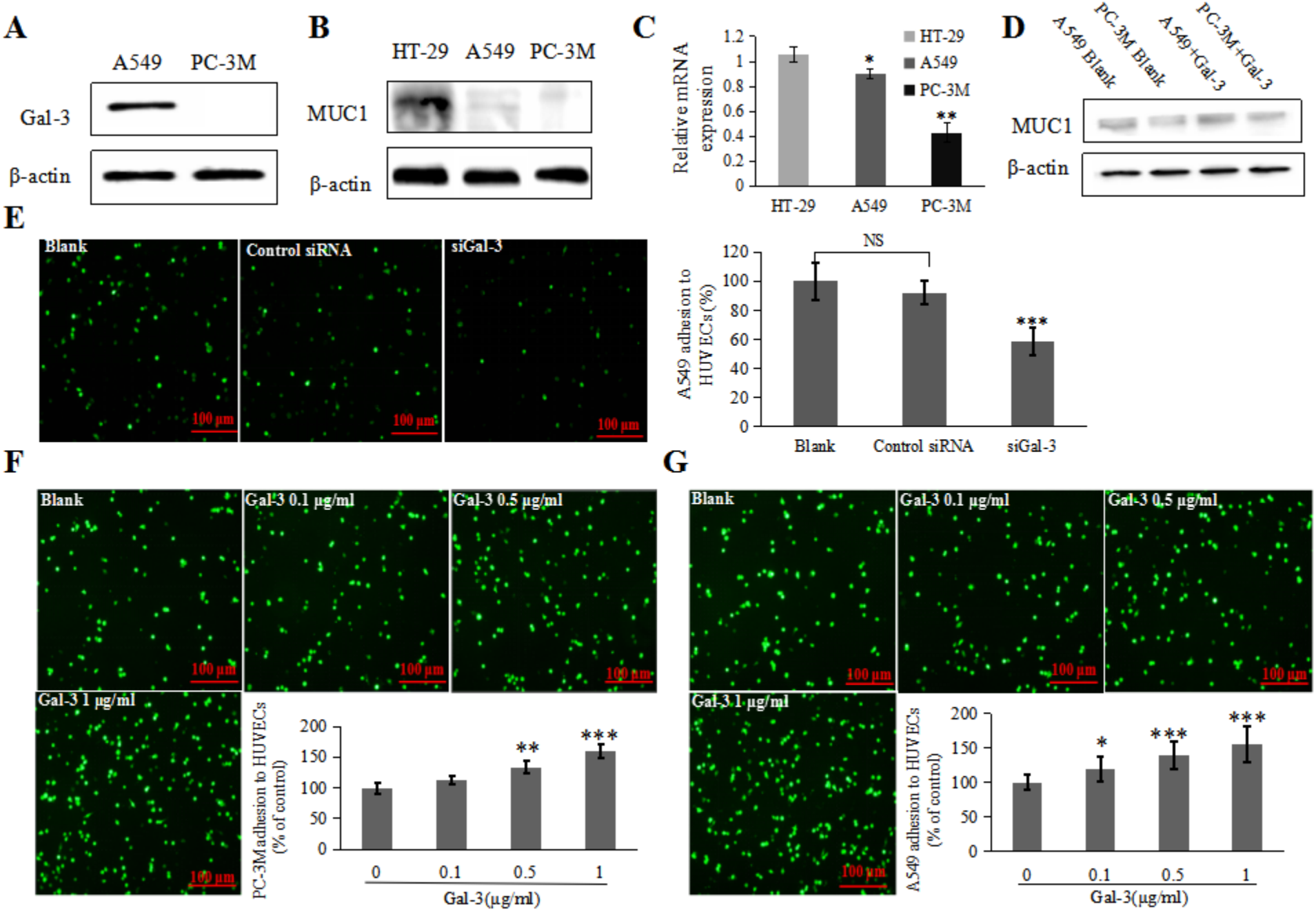
Figure 8. Exogenous Gal-3 promoted tumor cell-HUVEC adhesion by upregulation of the expression of N-cadherin and CD44 in cells of N-cadherin and CD44 suppression. A and B: The effect of exogenous Gal-3 on A549 cell adhesion to HUVECs after siRNA suppression of N-cadherin (A) and CD44 (B). C: A549 cells were treated with 1 μg/ml recombinant Gal-3 for 8 h after transfection of the cells with N-cadherin siRNA or CD44 siRNA. The expression of N-cadherin and CD44 were determined by Western blotting. Data are presented as the means ± S.E. from three separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

Figure 9. Effect of cell-associated Gal-3 on the migration and invasion of A549 cells. Effects of cell-associated Gal-3 on the migration and invasion of A549 cells in the presence or absence of 10 μmol/L ICG-001 were determined using wound scratch assay (A) or transwell invasion assay (B). C: Effects of ICG-001 on the expression of MMP-2 and MMP-9 were evaluated by Western blotting. Data are presented as the

means ± S.E. from three separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, *vs*. control siRNA.

Figure 10. Downregulation of Gal-3 inhibited pumonary metastasis of A549 cells in nude mice. A, B and C: A549-Luc cells which were transfected with siGal-3 or control siRNA were injected into nude mice *via* tail vein to observe lung metastasis in IVIS Imaging system at day 10, 20, 40. D. Macroscopic appearance of lungs at day 40 after tumor cell injections. Arrows, metastatic foci.

Figure 1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A549
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Gal-3
β-actin
B
HT-29
A549
PC-3M
MUC1
β-actin
C
Relative mRNA expression
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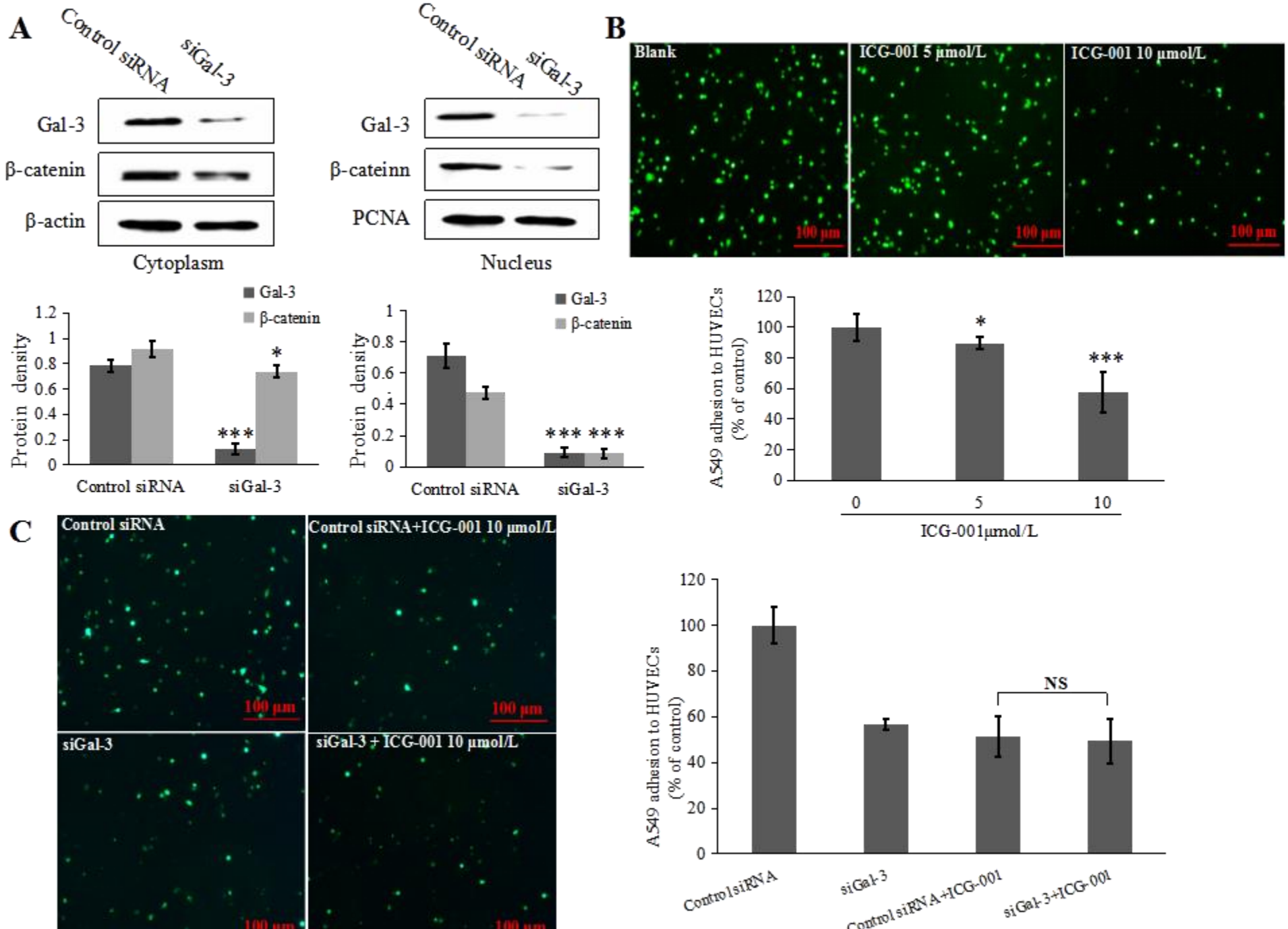
1
0.8
0.6
0.4
0.2
0
HT-29
A549
PC-3M
*
**
HT-29
A549
PC-3M
D
A549 Blank
PC-3M Blank
A549+Gal-3
PC-3M+Gal-3
MUC1
β-actin
E
Blank
Control siRNA
siGal-3
100 μm
A549 adhesion to HUVECs (%)
120
100
80
60
40
20
0
NS
***
Blank
Control siRNA
siGal-3
F
Blank
Gal-3 0.1 μg/ml
Gal-3 0.5 μg/ml
Gal-3 1 μg/ml
100 μm
PC-3M adhesion to HUVECs (% of control)
200
150
100
50
0
**
***
0
0.1
0.5
1
Gal-3(μg/ml)
G
Blank
Gal-3 0.1 μg/ml
Gal-3 0.5 μg/ml
Gal-3 1 μg/ml
100 μm
A549 adhesion to HUVECs (% of control)
200
150
100
50
0
*
***
***
0
0.1
0.5
1
Gal-3(μg/ml)

Figure 2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Control siRNA
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Gal-3
β-catenin
β-actin
Cytoplasm
Control siRNA
siGal-3
Gal-3
β-cateinn
PCNA
Nucleus
Gal-3
β-catenin
Protein density
1.2
1
0.8
0.6
0.4
0.2
0
***
*
Control siRNA
siGal-3
Gal-3
β-catenin
Protein density
1
0.8
0.6
0.4
0.2
0
*** ***
Control siRNA
siGal-3
B
Blank
ICG-001 5 μmol/L
ICG-001 10 μmol/L
100 μm
100 μm
100 μm
A549 adhesion to HUVECs (% of control)
120
100
80
60
40
20
0
*
***
0
5
10
ICG-001μmol/L
C
Control siRNA
Control siRNA+ICG-001 10 μmol/L
siGal-3
siGal-3 + ICG-001 10 μmol/L
100 μm
100 μm
100 μm
100 μm
A549 adhesion to HUVECs (% of control)
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100
80
60
40
20
0
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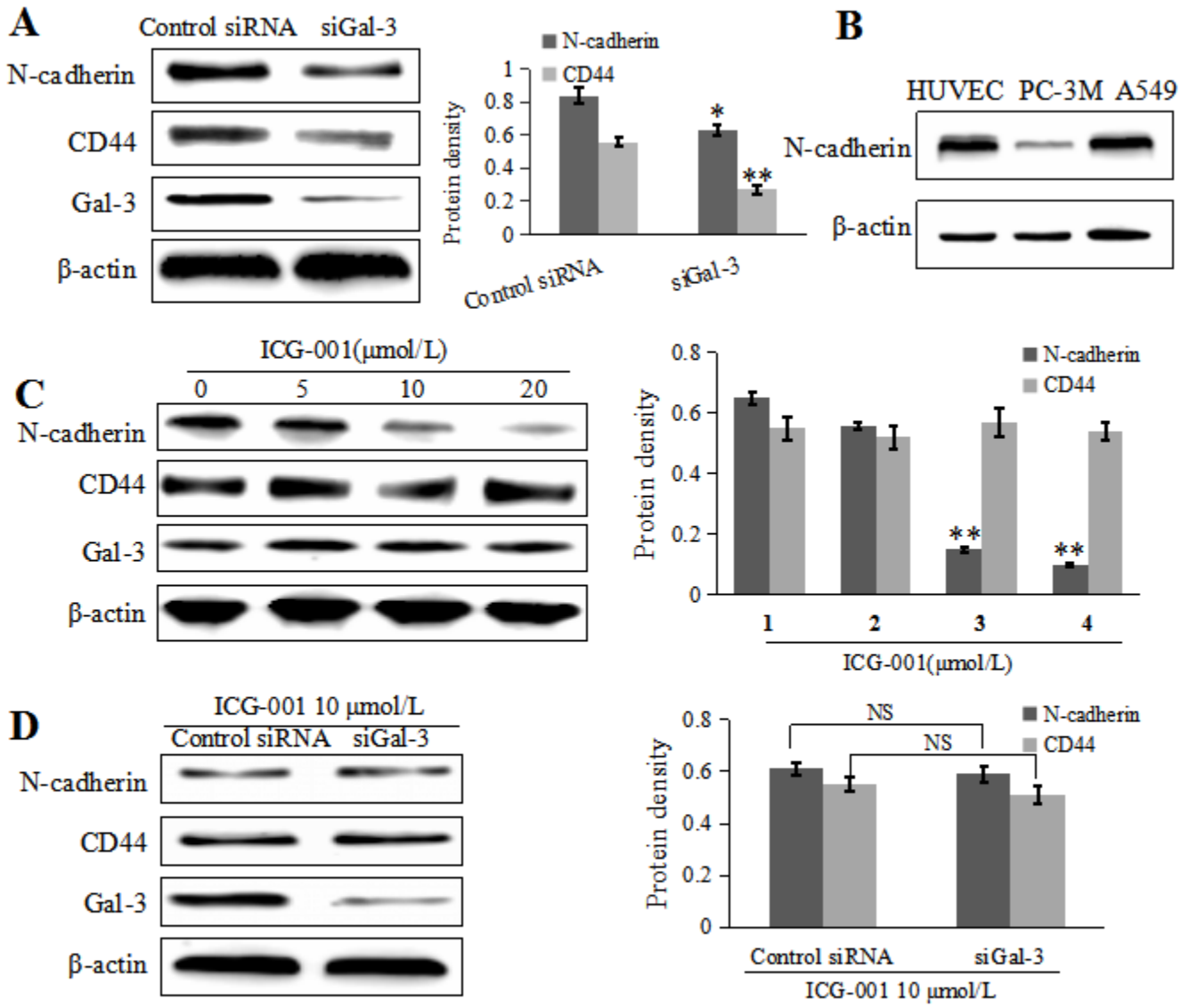
Control siRNA
siGal-3
Control siRNA+ICG-001
siGal-3+ICG-001

Figure 3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CD44
Gal-3
β-actin
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CD44
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0.6
0.4
0.2
0
*
**
Control siRNA
siGal-3
B
HUVEC
PC-3M
A549
N-cadherin
β-actin
C
ICG-001(μmol/L)
0
5
10
20
N-cadherin
CD44
Gal-3
β-actin
N-cadherin
CD44
Protein density
0.8
0.6
0.4
0.2
0
**
**
1
2
3
4
ICG-001(μmol/L)
D
ICG-001 10 μmol/L
Control siRNA
siGal-3
N-cadherin
CD44
Gal-3
β-actin
NS
NS
N-cadherin
CD44
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0.4
0.2
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Control siRNA
siGal-3
ICG-001 10 μmol/L

Figure 4

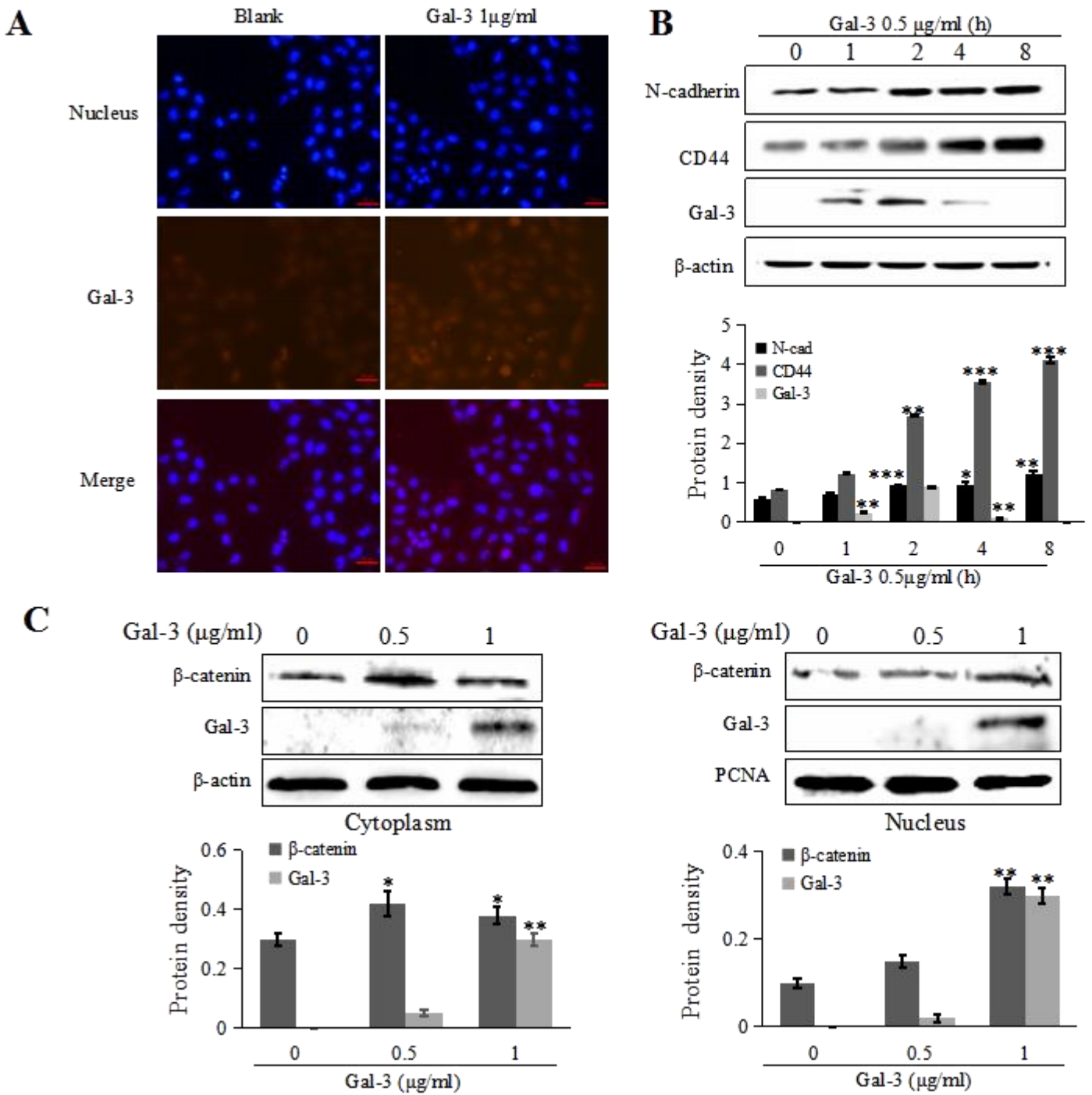
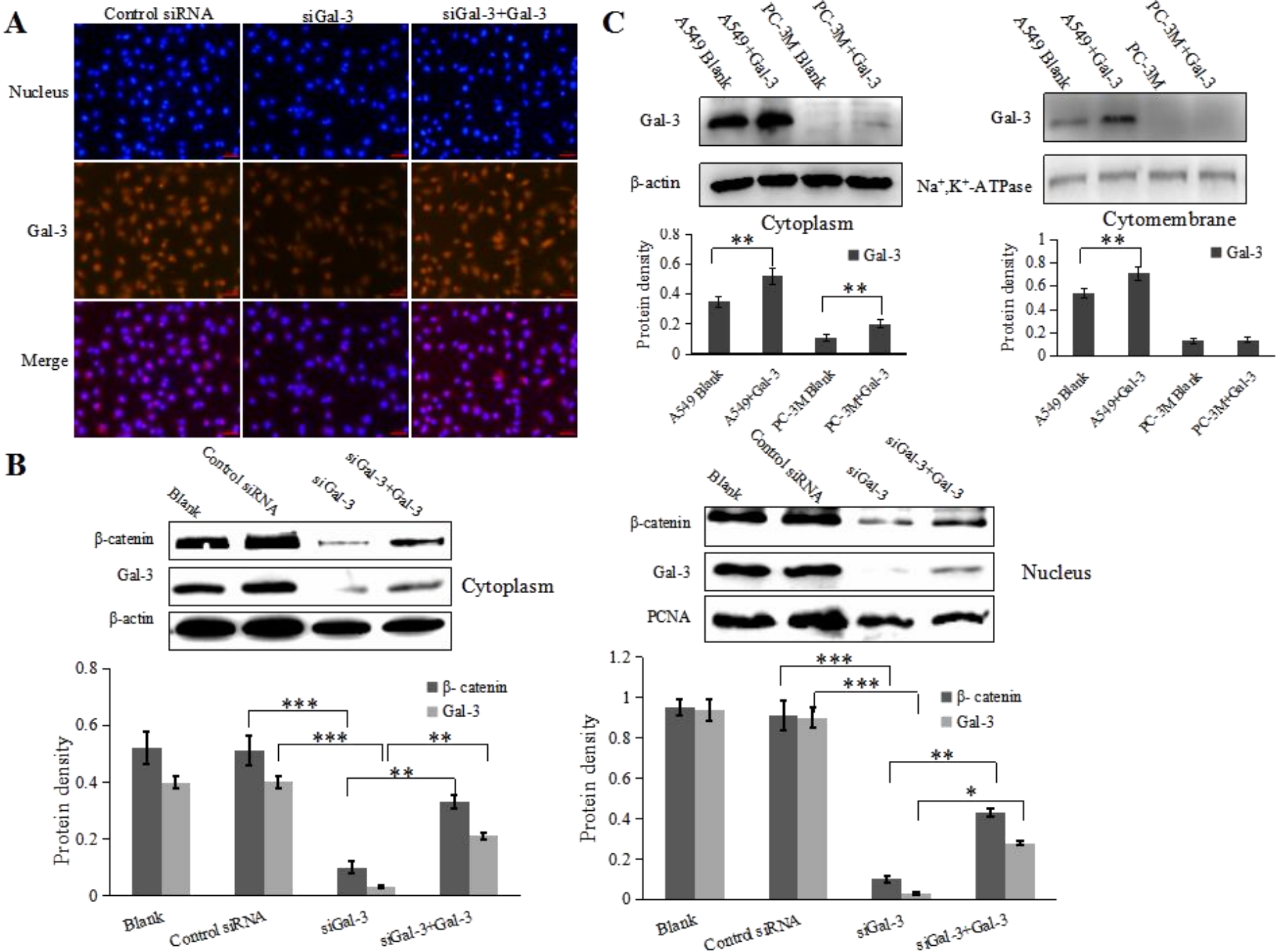

A
Blank
Gal-3 1μg/ml
Nucleus
Gal-3
Merge
B
Gal-3 0.5 μg/ml (h)
0 1 2 4 8
N-cadherin
CD44
Gal-3
β-actin
Protein density
N-cad
CD44
Gal-3
Gal-3 0.5μg/ml (h)
C
Gal-3 (μg/ml) 0 0.5 1
β-catenin
Gal-3
β-actin
Cytoplasm
Protein density
β-catenin
Gal-3
Gal-3 (μg/ml)
Gal-3 (μg/ml) 0 0.5 1
β-catenin
Gal-3
PCNA
Nucleus
Protein density
β-catenin
Gal-3
Gal-3 (μg/ml)

Figure 5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Nucleus
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Control siRNA
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β-actin
Cytoplasm
Protein density
β- catenin
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siGal-3+Gal-3
C
A549 Blank
A549+Gal-3
PC-3M Blank
PC-3M+Gal-3
Gal-3
β-actin
Cytoplasm
Protein density
Gal-3
A549 Blank
A549+Gal-3
PC-3M
PC-3M+Gal-3
Gal-3
Na+,K+-ATPase
Cytomembrane
Protein density
Gal-3
Blank
Control siRNA
siGal-3
siGal-3+Gal-3
β-catenin
Gal-3
PCNA
Nucleus
Protein density
β- catenin
Gal-3
Blank
Control siRNA
siGal-3
siGal-3+Gal-3

Figure 6

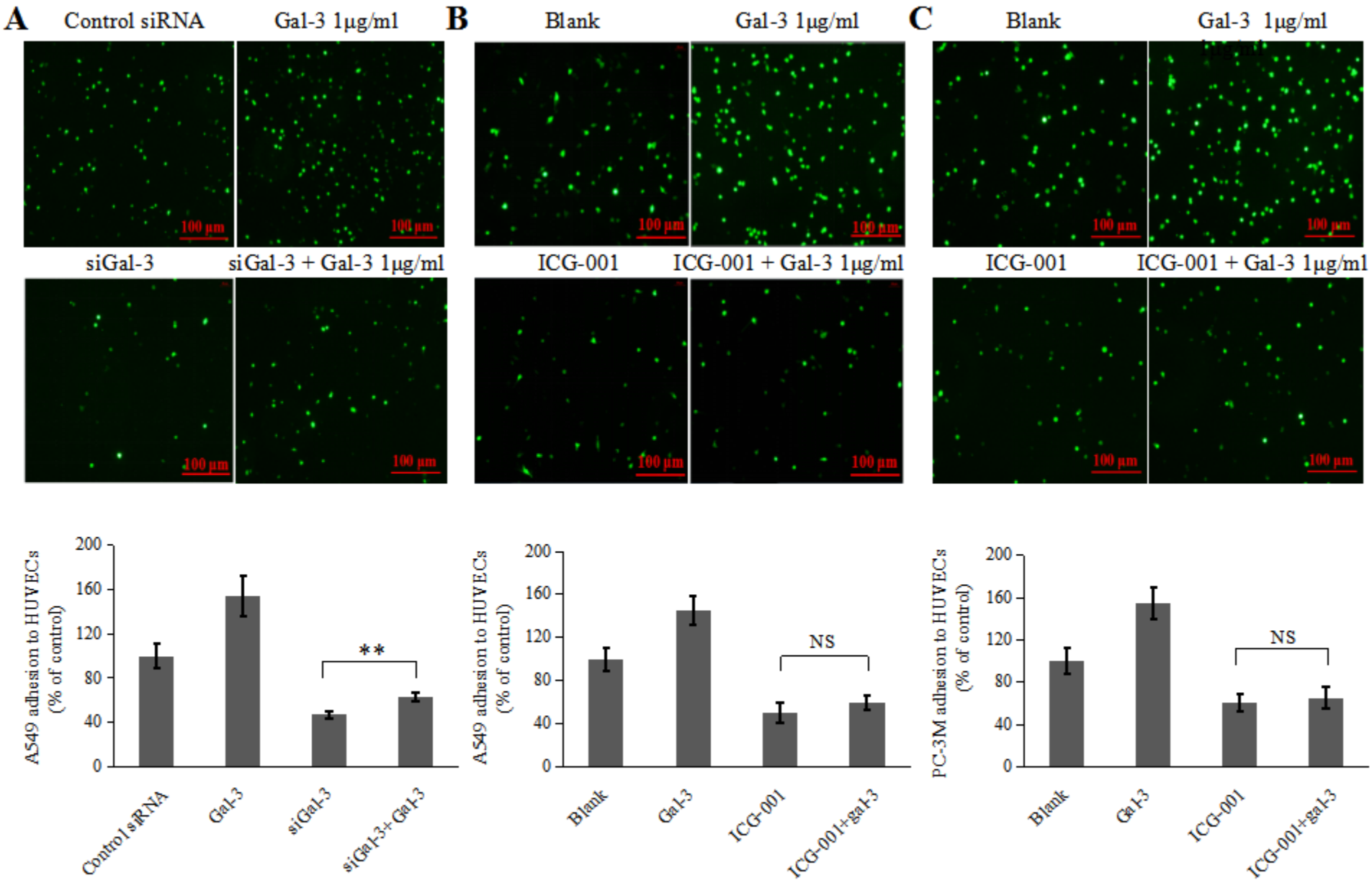

A
Control siRNA
Gal-3 1μg/ml
siGal-3
siGal-3 + Gal-3 1μg/ml
B
Blank
Gal-3 1μg/ml
ICG-001
ICG-001 + Gal-3 1μg/ml
C
Blank
Gal-3 1μg/ml
ICG-001
ICG-001 + Gal-3 1μg/ml
100 μm
A549 adhesion to HUVECs (% of control)
200
160
120
80
40
0
**
Control siRNA
Gal-3
siGal-3
siGal-3+Gal-3
A549 adhesion to HUVECs (% of control)
NS
Blank
Gal-3
ICG-001
ICG-001+gal-3
PC-3M adhesion to HUVECs (% of control)
NS
Blank
Gal-3
ICG-001
ICG-001+gal-3

Figure 7

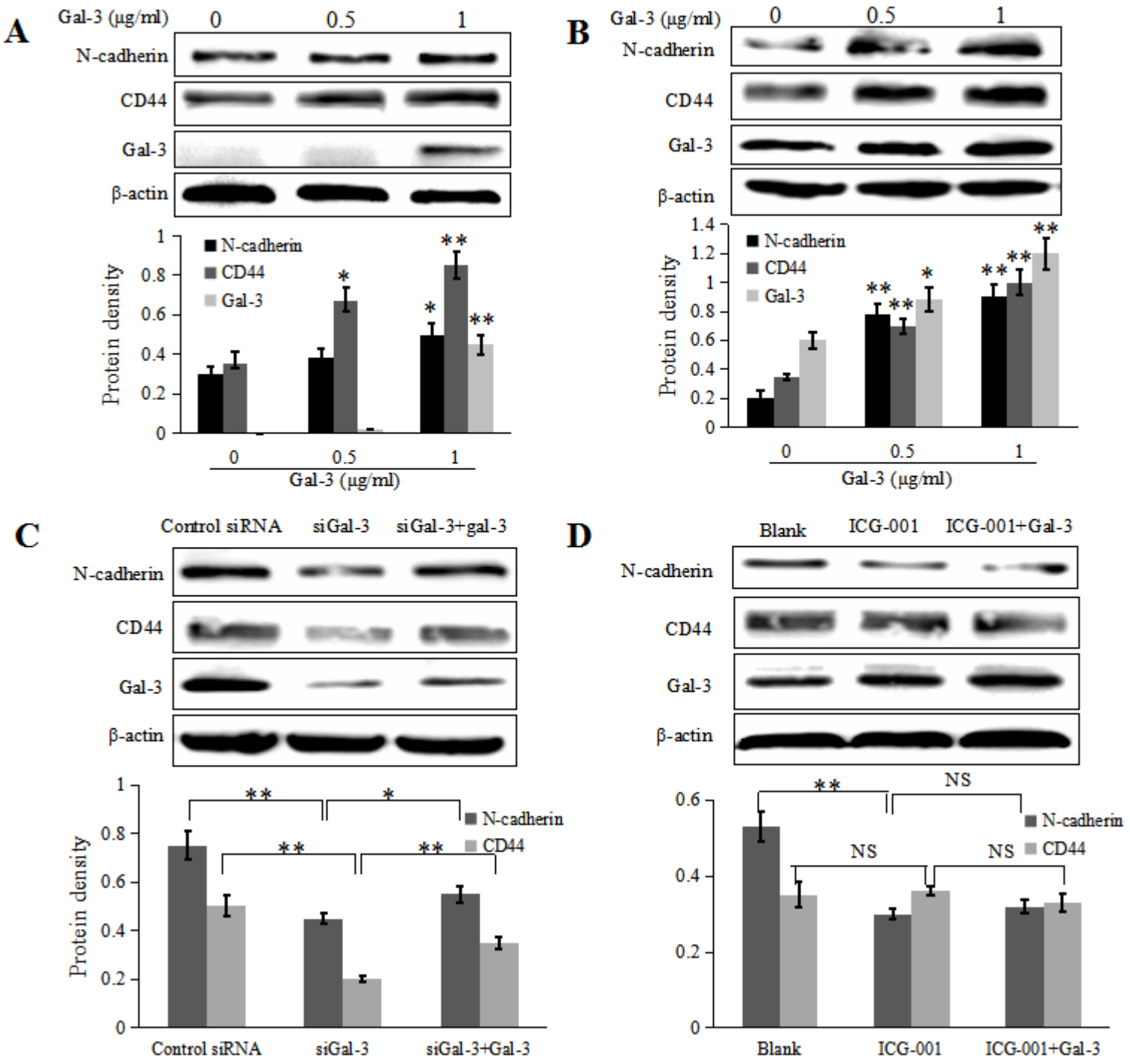

A
Gal-3 (μg/ml)
0
0.5
1
N-cadherin
CD44
Gal-3
β-actin
Protein density
N-cadherin
CD44
Gal-3
Gal-3 (μg/ml)
B
C
Control siRNA
siGal-3
siGal-3+gal-3
D
Blank
ICG-001
ICG-001+Gal-3
NS

Figure 8

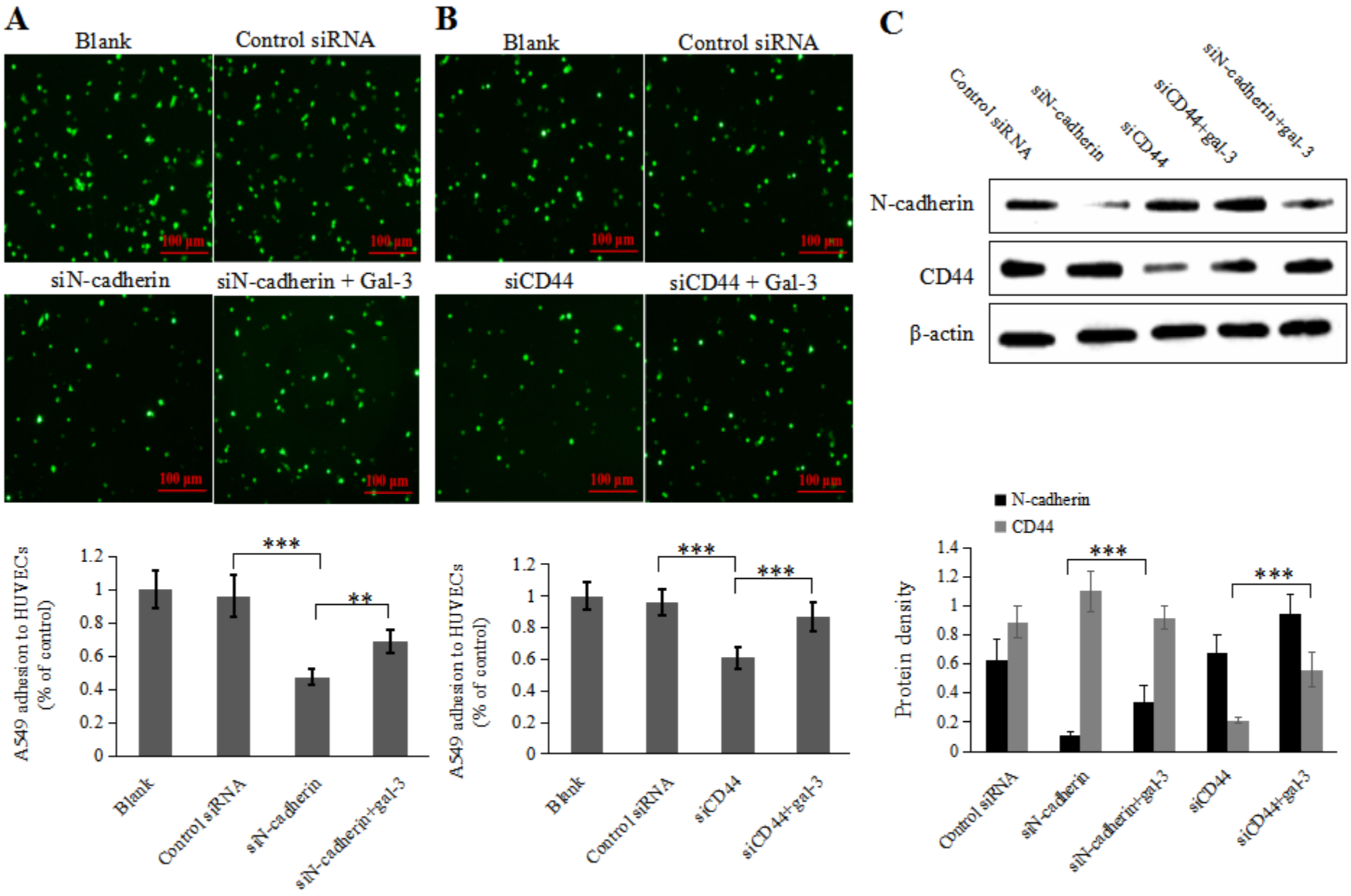

A
Blank
Control siRNA
siN-cadherin
siN-cadherin + Gal-3
100 μm
B
Blank
Control siRNA
siCD44
siCD44 + Gal-3
100 μm
C
Control siRNA
siN-cadherin
siCD44
siCD44+gal-3
siN-cadherin+gal-3
N-cadherin
CD44
β-actin
A549 adhesion to HUVECs (% of control)
***
**
Blank
Control siRNA
siN-cadherin
siN-cadherin+gal-3
A549 adhesion to HUVECs (% of control)
***
***
Blank
Control siRNA
siCD44
siCD44+gal-3
N-cadherin
CD44
Protein density
***
***
Control siRNA
siN-cadherin
siN-cadherin+gal-3
siCD44
siCD44+gal-3

Figure 9

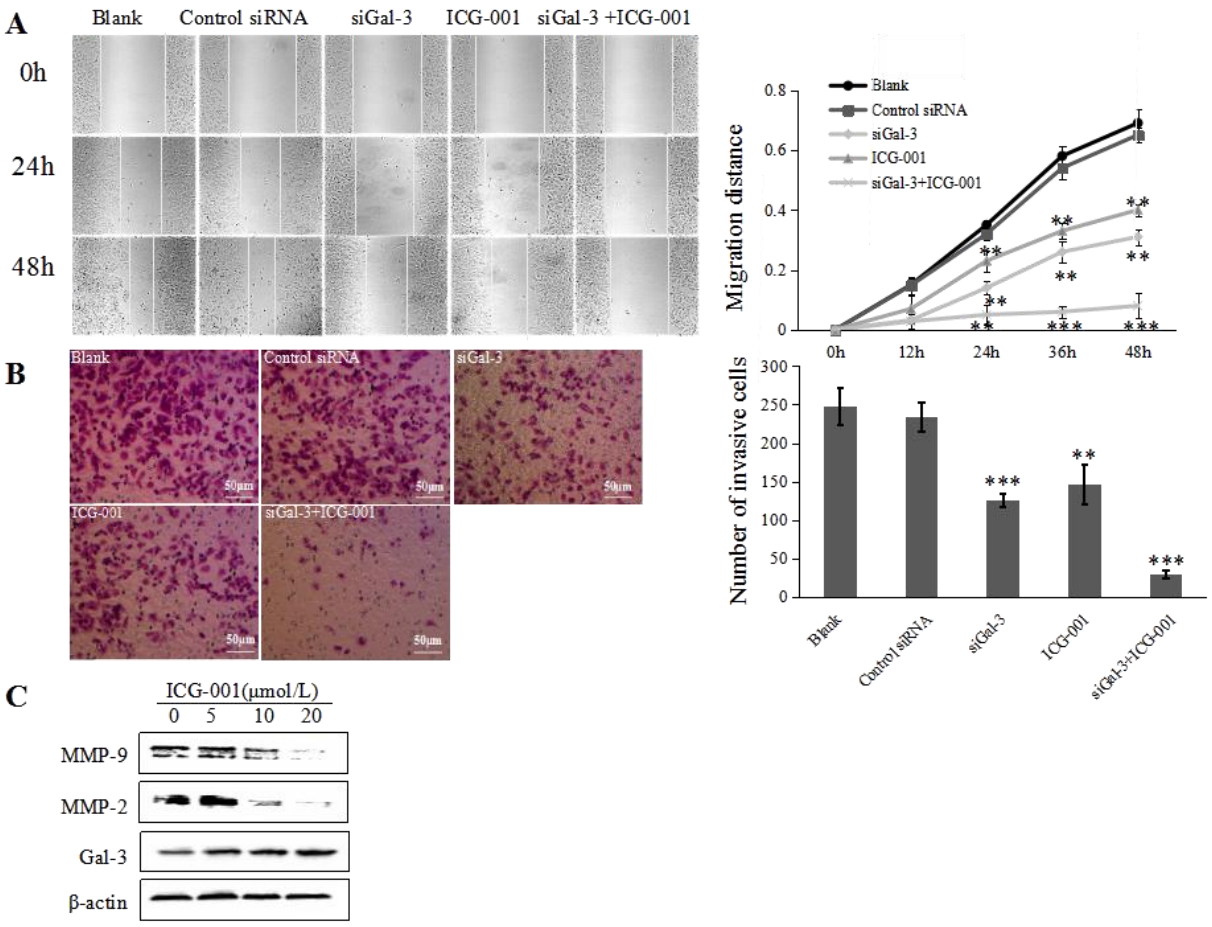
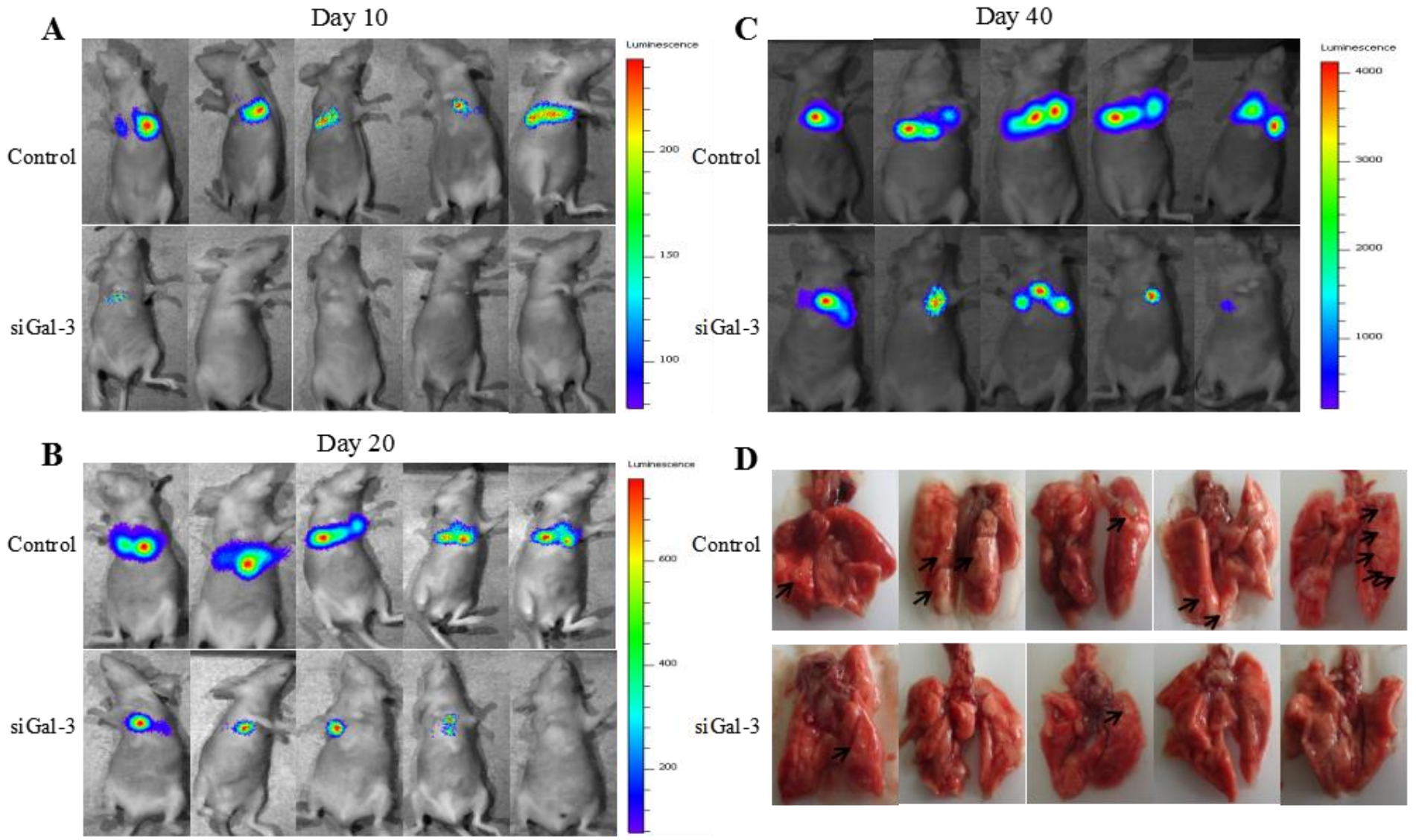

A
Blank
Control siRNA
siGal-3
ICG-001
siGal-3 +ICG-001
0h
24h
48h
B
Blank
Control siRNA
siGal-3
ICG-001
siGal-3+ICG-001
50μm
C
ICG-001(μmol/L)
0 5 10 20
MMP-9
MMP-2
Gal-3
β-actin
Migration distance
Blank
Control siRNA
siGal-3
ICG-001
siGal-3+ICG-001
0h 12h 24h 36h 48h
Number of invasive cells
Blank
Control siRNA
siGal-3
ICG-001
siGal-3+ICG-001

Figure 10


A
Day 10
Control
siGal-3
Luminescence
200
150
100
B
Day 20
Control
siGal-3
Luminescence
600
400
200
C
Day 40
Control
siGal-3
Luminescence
4000
3000
2000
1000
D
Control
siGal-3